REMARKS

Claims 3-55 have been canceled by the Amendment. Thus, the pending claims are 1, 2, 56 and 57.

Claim 1 has been amended to recite testing for the remission stage of atopic dermatitis in "a test subject having atopic dermatitis." Claim 1 has also been amended to recite comparing the expression level measured with "the expression level of the NOR-1 gene in the eosinophil cells of the test subject during the exacerbation stage of atopic dermatitis." Support for these amendments can be found in Example 1 of the Specification and, in particular, at page 36, lines 21-22 and Table 5.

Claim 56 has been amended to recite assessing the effect of a therapy on "an individual in the exacerbation stage of atopic dermatitis." Claim 56 has also been amended to recite "determining whether the test subject has had a decrease in eosinophil cell number after the therapy." Claim 56 has further been amended to recite that the increase in NOR-1 gene expression level and "a decrease in eosinophil cell number" after the therapy indicates "that the therapy has been effective." Support for these amendments can be found in the Specification in Example 1 and, in particular, at page 43, line 7 through page 44, line 20 and Tables 2 and 5.

No new matter has been added. Further remarks are set forth below.

Rejection of Claims 1, 2 and 54-57 Under 35 U.S.C. § 112, First Paragraph

Claims 1, 2 and 54-57 have been rejected under 35 U.S.C. § 112, first paragraph for failing to comply with the enablement requirement. The Examiner states that the claims contain subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention and that, without further guidance, a person of skill in the art could not use the invention as claimed without undue experimentation. Specifically, the Examiner asserts that the increase in NOR-1 gene expression could be due to treatment with drugs rather than due to entry of the subjects into the remission stage and further alleges that without data on the expression of the NOR-1 gene in those individuals treated with drugs who were healthy or who were in the exacerbation stage yet unresponsive to the drugs, that it is not predictable whether NOR-1 gene expression could be used to accurately diagnose the remission stage of atopic dermatitis without undue experimentation. In addition, the Examiner states that the term "test subject" in claims 1, 2, 54 and 55 encompasses any subject and that Applicants' results are not enabled for taking

measurements in a test subject who is healthy or in the exacerbation stage of atopic dermatitis, nor for comparing NOR-1 expression levels in different individuals. The Examiner also states that claims 56 and 57 lack enablement for an individual with atopic dermatitis who is already in the remission stage.

The Examiner acknowledges that he "does not dispute the decrease in eosinophil number and increase in NOR-1 gene expression observed in four out of seven of the atopic dermatitis patients in the remission stage, as compared to the exacerbation stage." (See Office Action 11/18/2005 at page 5, lines 17-19). The Examiner further notes that, with respect to claims 1, 2, 54 and 55, that Applicants' results would provide enablement for the recited methods if the test subject and exacerbation subject were the same individual, the Examiner stating that Applicants' would be enabled for "a method of testing for the remission stage of atopic dermatitis comprising measuring NOR-1 gene expression in a subject in the exacerbation stage of atopic dermatitis, treating the subject, measuring NOR-1 again, and comparing the two test results, wherein the remission stage of atopic dermatitis associated with a decrease in eosinophils cell number is indicated by an increase in NOR-1 gene expression in the second measurement." (See Office Action 11/18/2005 at page 6, lines 9-20). Further, the Examiner states that, with respect to claims 56 and 57, drawn to assessing the effect of a therapy on the atopic dermatitis of an individual, the claims are enabled if an increase in NOR-1 gene expression "indicates that the individual in the exacerbation stage has entered the remission stage associated with a decrease in the number of eosinophil cells." (See Office Action 11/18/2005 at page 7, lines 19-27).

In order to make a rejection based on enablement, the Examiner has the burden to establish a reasonable basis to question the enablement provided for the claimed invention. *In re Wright*, 999 F.2d 1557, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993) (emphasis added). As stated by the Federal Circuit Court "it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure." 439 F.2d at 224, 169 USPQ at 370.

In this case, the Examiner's basis for questioning enablement of the claimed method (i.e., that there is no data showing the level of NOR-1 gene expression in individuals who were treated

with drugs but did not enter the remission stage of atopic dermatitis) is not a reasonable basis to question the objective truth of Applicants' disclosure. In fact, the Examiner's assertion that, without this data, the increase in NOR-1 gene expression could be due to drug treatment instead of due to the subjects' entry into the remission stage of atopic dermatitis is pure speculation. Tables 2 and 5 provide measurements for individuals with atopic dermatitis who were treated with drugs and entered a remission stage associated with a decrease in eosinophil cell numbers (i.e., patients 1, 2, 3 and 5). The NOR-1 gene expression levels for patients 1, 3 and 5 increased dramatically when the patients entered the remission stage associated with a decrease in eosinophil cell number, and this increase in NOR-1 gene expression was confirmed by statistical analysis. Contrary to the Examiner's statement, Applicants have disclosed corresponding data for individuals in the exacerbation stage of atopic dermatitis who were less responsive to drug treatment. Specifically, Tables 2 and 5 also provide measurements for individuals who were treated with drugs *but did not enter the remission stage associated with a decrease in eosinophil cell number* (i.e., patients 4, 6 and 7). In contrast to patients treated with drugs who entered the remission stage of atopic dermatitis associated with a decrease in eosinophil cell number, NOR-1 expression in patients 4 and 6 greatly decreased while there was only a small change in NOR-1 expression in patient 7. Faced with the aforementioned data, one having skill in the art would not have concluded that the drug treatment caused a change in NOR-1 expression. Instead, based on his knowledge of the art, the skilled artisan would have interpreted the data to have shown that an increase in NOR-1 expression and a decrease in eosinophil cell number were associated with the transition of patients into the remission stage of atopic dermatitis.

Moreover, the Examiner's assertion that drug treatment alone could have caused the increase in NOR-1 gene expression has been disproved in Kagaya S *et al.*, *Int Arch Allergy Immunol*, 137(suppl 1):35-44, 2005 (hereinafter "Kagaya *et al.*"), a copy of which is attached as Exhibit A. Kagaya *et al.* teaches that NOR-1 gene expression is in fact unchanged due to drug (i.e., steroid) treatment. Thus, Kagaya *et al.* demonstrates that steroids, given as an endemic liniment, do not affect the expression of the NOR-1 gene (see at page 42, cols. 1-2).

Accordingly, based on the data disclosed in the Specification and his knowledge of the art, one having skill in the art would have concluded that drug treatment alone did not cause an increase in NOR-1 gene expression and, consequently, could have practiced the claimed methods in a predictable manner. Thus, the Specification enables one of skill in the art to practice the claimed invention without undue experimentation.

Claims 54 and 55 have been canceled, thereby obviating the rejection with respect to those claims. Claim 1 has been amended to recite "a test subject having atopic dermatitis" to better define the test subjects the method encompasses. Claim 1 has also been amended to recite comparing the expression level of NOR-1 measured in the test subject having atopic dermatitis to that in the eosinophil cells of "the test subject during the exacerbation stage of atopic dermatitis" such that the test subject and exacerbation subject in the claimed method are the same individual. Thus, as amended, Claim 1 and dependent Claim 2 meet the enablement requirement of 35 U.S.C. § 112, first paragraph.

Further, Claim 56 has been amended to recite assessing the effect of a therapy on an individual "in the exacerbation stage of atopic dermatitis" in order to better define those individuals encompassed by the method. In addition, to better define the method, Claim 56 has been amended to include a step of "determining whether the test subject has had a decrease in eosinophil cell number after the therapy" and recite that an increase in NOR-1 gene expression and "a decrease in eosinophil cell number" after the therapy indicate that "the therapy has been effective." Therefore, amended Claim 56 and dependent Claim 57 satisfy the enablement requirement under 35 U.S.C. § 112, first paragraph.

Accordingly, as amended, Claims 1, 2, 56 and 57 of the subject application are fully enabled by the Specification such that one having skill in the art, based on the application disclosure and his knowledge of the art, could practice the claimed invention without undue experimentation. Reconsideration and withdrawal of the rejection are requested.

Information Disclosure Statement

As requested by the Examiner, a clean copy of the PTO-1449 form enclosed with the Information Disclosure Statement (IDS) filed on 6/7/2004 is being provided so that the Examiner can indicate consideration of references AM, AO and AQ for the application filewrapper.

CONCLUSION

In view of the above amendments and remarks, it is believed that all claims are in condition for allowance, and it is respectfully requested that the application be passed to issue. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned.

Respectfully submitted,

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NR4A Orphan Nuclear Receptor Family in Peripheral Blood Eosinophils from Patients with Atopic Dermatitis and Apoptotic Eosinophils in vitro

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Key Words
Apoptosis · Atopic dermatitis · CD30 · Eosinophil · Gene expression · GeneChip · NR4A nuclear receptor family · NOR1 · Nur77 · Nurr1 · Real-time RT-PCR

Abstract
To identify novel genes related to the clinical signs of atopic dermatitis (AD), differentially expressed genes were sought in peripheral blood eosinophils from both AD patients and healthy volunteers. RNA was prepared from eosinophils, expression of various genes was monitored using the Affymetrix GeneChip, and expression was quantified by real-time RT-PCR. Two genes, Nur77 and NOR1, members of NR4A orphan nuclear receptor family, were expressed at a significantly higher level in AD patients than in healthy volunteers. Expression of another gene in the NR4A receptor family, Nurr1, was also higher in AD patients than in healthy volunteers. When peripheral blood leukocytes from healthy volunteers were fractionated, NOR1 expression was highest in eosinophils, but expression of Nur77 and Nurr1 genes was not eosinophil-specific. Extremely intense apoptosis was induced in both eosinophils and an eosinophil cell line, AML14.3D10, by treatment with antibody (Ab) to both CD30 and Fas. Rapid expression of the genes for the NR4A receptor family was observed with anti-CD30 Ab treatment but not with anti-Fas Ab. The NR4A orphan nuclear receptor family gene expression and the subsequent eosinophil apoptosis were downregulated by the MAPK inhibitor, U0126. These results suggest that the expression of the NR4A receptor family genes through CD30 signaling may regulate eosinophil apoptosis in allergic conditions such as AD.

Introduction

While both multigene interactions and environmental factors are likely to contribute to allergic diseases, eosinophils play causative roles in conditions such as asthma [1–3] and atopic dermatitis (AD) [4, 5], and are involved

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in tissue damage during the chronic phase of allergic diseases. An understanding of gene expression in eosinophils would be helpful to explain the pathogenesis of allergic diseases. Recent improvements in the technology of comprehensive gene expression analysis, such as differential display [6, 7], DNA microarray [8, 9], and real-time RT-PCR [10, 11], make possible the efficient survey of the expression of large numbers of genes in clinical samples.

We compared gene expression in peripheral blood eosinophils from both AD patients and healthy volunteers and identified some genes that were differentially expressed [12]. Some of the differentially expressed genes belonged to the human orphan nuclear receptor family, NR4A. The NR4A family consists of 3 genes, Nur77, Nurr1, and NOR1 that encode ligand-unknown transcription factors. The family regulates gene expression that is involved in reproduction, development, and adult physiology, and they have been implicated in diverse cellular signaling events, such as neuroendocrine regulation [13–15], neural differentiation [16, 17], and liver regeneration [18]. Nur77 and NOR1 also play a key role in apoptosis of T lymphocytes [19–22] as well as other lymphoid cell types. We have extended our earlier investigations to clarify the relationship between eosinophil-specific apoptosis that occurs via the CD30 pathway [23] and expression of the NR4A family genes in vitro. The importance of NR4A orphan nuclear receptor family gene expression in eosinophils to the pathology of allergic conditions was also studied.

Materials and Methods

Blood Samples for GeneChip and Real-Time RT-PCR

Blood samples from 2 patients with severe AD, 2 patients with mild AD, and 2 healthy volunteers were used for Affymetrix GeneChip analysis. The gene expression in the patient population shown in table 1 was confirmed by real-time RT-PCR. Patients with AD were diagnosed according to the criteria of Hanifin [24], and the severity of AD was judged using a modified version of Leicester's scoring system, as reported previously [25, 26]. Briefly, five clinical features (erythema, papule, excoriation, oozing, and lichenification) were evaluated at six body sites (hand, trunk, elbow, hand, knee, and foot). The percentage area of clinical features per whole body surface was used to define the AD severity (mild <10%; moderate >10% and <30%; severe >30%).

The AD patients were treated with topical glucocorticoid ointments. A mild steroid (mainly hydrocortisone acetate) was used for treating the face and a strong steroid (mainly dexamethasone valerate) for the body. None of the AD patients had been treated with systemic glucocorticoids. According to the diagnostic standards of Hanifin [24], we diagnosed characteristic eczema when the diseased areas appeared on both face and chest. Chronic and recurrent dermatitis was also characterized in patients less than 1 year old by a duration of more than 2 months. Moreover, all patients under 1 year of age who were selected for study were IgE-positive and had case histories of dry skin and staphylococcal infection. In cases aged over 1 year, persistence of the diseased area for more than 6 months was a diagnostic criterion.

Table 1. Patient population

Healthy controls	13 persons (6 males, 7 females) age 26–63 years (average 39.3)
Mild AD	15 persons (9 males, 6 females) age 0–21 years (average 10.2)
Moderate AD	15 persons (11 males, 4 females) age 3–18 years (average 9.4)
Severe AD	18 persons (9 males, 9 females) age 0–29 years (average 11.3)

Written informed consent to participate in the study was obtained from volunteers or parents of minors after providing them with detailed information about the study and the rights of subjects. Our experiments were authorized by the ethical committees of both Genox Research and the National Research Institute for Child Health and Development.

Purification of Peripheral Blood Leukocytes

Granulocytes were isolated from heparinized venous blood by Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) centrifugation. Red blood cells were removed by hypotonic lysis. The CD16-negative flow-through eosinophil fractions were collected with MACS separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany). The eosinophil cell purity was always greater than 99% by Diff-Quick staining. The CD16-positive fractions were collected as neutrophils. The T cell, B cell, and monocyte fractions were isolated from the peripheral blood mononuclear cell layer by using CD3 and CD14 MACS separation kits and columns (Miltenyi Biotec). Total RNA from both isolated fractions and cultured cells was prepared using an RNA extraction kit (Isogen solution, Nippon Gene, Tokyo, Japan).

Culture of Peripheral Blood Eosinophils after Stimulation with Anti-CD30 Antibody or Anti-Fas Antibodies and Evaluation of Postculture Cell Apoptosis/Viability

Eosinophil stimulation with anti-CD30 antibody (Ab) was done as previously described [23]. Eosinophils with a purity of more than 99% isolated from a healthy volunteer were suspended at a cell density of 1×10^6/ml in Iscove's minimal essential medium (IMEM) supplemented with 10% heat-inactivated fetal calf serum, 5×10^{-5} *M* 2-mercaptoethanol, and 1 ng/ml human recombinant IL-5 (R&D Systems, Minneapolis, Minn., USA). For culture experiments, 6-well culture plates were coated overnight at 4°C with 1.5-ml aliquots of anti-CD30 Ab (10 μg/ml final concentration; Ber-H8, IgG1; BD Pharmingen, San Diego, Calif., USA) or anti-Fas Ab (1 μg/ml final concentration; clone CH-11, IgM; Medical Biological Laboratories, Gunma, Japan). Nonspecific binding to wells was blocked by incubation with 2-ml aliquots of PBS containing

1% human serum albumin (heat-denatured at 65°C for 1 h) for at least 2 h at room temperature. The wells were washed twice with prewarmed IMEM before use.

Cell viability was tested with an apoptosis detection kit (MEBCYTO-Apoptosis kit; Medical Biological Laboratories, Nagoya, Japan) using FITC-conjugated annexin V [27] and propidium iodide (PI) according to the manufacturer's instructions. Briefly, cultured eosinophils were harvested at various time points by gentle pipetting, followed by a single wash with PBS. The harvested eosinophils were suspended on Ca^{2+}-containing buffer and incubated with FITC-conjugated annexin V and 10 μg/ml PI for 15 min at room temperature. The annexin V-positive and PI-stained cells were quantified by flow cytometer (FACScan analyzer; BD Biosciences, San Jose, Calif., USA).

Culture of Eosinophil Cell Line, AML14.3D10, after Stimulation with Anti-CD30 or Anti-Fas Abs and Evaluation of Postculture Cell Apoptosis/Viability

A human eosinophil cell line, AML14.3D10 cells [28], obtained from Wright State University (Dayton, Ohio, USA), were grown in RPMI 1640 containing 10% heat-inactivated fetal calf serum, 5×10^{-5} *M* 2-mercaptoethanol, and 1 m*M* sodium pyruvate. The soluble anti-CD30 Ab (Ber-H8, IgG1) 10 μg/ml or anti-Fas Ab (clone CH-11, IgM) 1 μg/ml were added to 0.5×10^6/ml of the AML14.3D10 cell suspension. Apoptosis and cell viability were measured using the same procedures described for peripheral blood eosinophils. U0126 (10 μ*M* final concentration; Calbiochem, San Diego, Calif., USA) or SB203508 (10 μ*M* final concentration; Calbiochem) was added simultaneously with the Abs and was present for the duration of the survival assay.

GeneChip Probes and Analysis

GeneChip analysis of 6 human peripheral blood eosinophils isolated from 2 severe AD patients, 2 mild AD patients, and 2 healthy volunteers was done as follows. The comparisons of eosinophils from the three groups of patients were done pairwise to give a total of four sets of comparisons for analysis. Genes showing greater than a 3-fold change (the ratio of values of average difference in the 2 samples being compared) in any of the comparison pairs were selected for further evaluation. The GeneChip Human Genome HG-U95A arrays (Affymetrix, Santa Clara, Calif., USA) with probes for 12,626 human genes were used to survey and quantify the level of mRNA transcripts. The manufacturer's instructions were followed for all procedures associated with the GeneChip array analysis. After scanning with the HP Gene Array Scanner, the fluorescence intensity with each probe was quantified using the array data analysis program, GeneChip Analysis Suite 4.0 (Affymetrix). The level of each single mRNA was determined as the average fluorescence intensity obtained by 16–20 paired (perfectly matched and single nucleotide-mismatched) primers consisting of 25-base oligonucleotides and shown as a value of average difference.

Real-Time RT-PCR

For quantitative confirmation of gene expression, the real-time quantitative RT-PCR method was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif., USA) as described previously [26, 29]. The total RNA samples were DNase treated, mixed with oligo (dT) 12–18 primer (Invitrogen, Carlsbad, Calif., USA), and converted to the first strand cDNA using superscript II reverse transcriptase (Invitrogen). The three nuclear receptor genes in the cDNA samples were amplified by introducing the forward and reverse primers and TaqMan probes designed by Primer Express software (Applied Biosystems): Nur77: 5'-CCA CTT TGG GAA GGA AGA TGC T, 5'-ACT TTC GGA TGA CCT CCA GAG A, and 5' 6-carboxy fluorescein (FAM)-ATG TAC AGC AGT TCT ACG ACC TGC TCT CCG-3' 6-carboxy-tetramethylrhodamine (TAMRA); Nurr1: 5'-AGC ACA GGC TAC GAC GTC AA, 5'-TCT TCT ACC TTA ATG GAG GAC TGC, and 5'FAM-TTG TAC CAA ATG CCC CTG TCC GGA-3' TAMRA; NOR1: 5'-TGG GTG CCC TGG TAG AAC T, 5'-GCT TCA GGT AGA AGA TGC GCT, and 5'FAM-AGG AAG ATC TGC ACC CTG GGC CTC-3' TAMRA.

The cDNA templates, including coding regions of these genes, were cloned from human leukocyte cDNA libraries and introduced into the system as standards. Expression levels of the human housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were also quantified in all samples using the standard primers and the TaqMan probe (PE Applied Biosystems).

Statistics

The nonparametric Dunnet signed rank test was used to evaluate the changes in gene expression in each group of clinical samples. The results were expressed as mean ± standard error (SE). p values of less than 0.05 were considered to be significant.

Results

Expression of NR4A Nuclear Receptor Family Genes in Peripheral Blood Eosinophils in vivo

Blood samples from 2 patients with severe AD, 2 patients with mild AD, and 2 healthy volunteers were used for Affymetrix GeneChip analysis. We selected 24 genes with more than 3-fold changed mRNA expression in eosinophils of 2 severe AD patients, 2 mild AD patients, and 2 healthy volunteers (table 2). Among these 24 genes, there were genes for two orphan nuclear receptors, Nur77 and NOR1, belonging to the NR4A nuclear receptor family. Nur77 was selected after a 4.5-fold increase in expression levels observed in samples from severe AD patients as compared to those from healthy individuals. NOR1 expression was 4.6 times higher in eosinophils from patients with severe AD as compared to those with mild AD. When large numbers of samples were used from the patients shown in table 1, the expression of genes for both receptors measured by real-time RT-PCR was significantly higher in eosinophils from AD patients than from healthy volunteers (fig. 1a, c). Nurr1, another receptor in the NR4A three-member nuclear receptor family, was not identified in GeneChip comparison analysis, but also showed a higher expression in eosinophils from AD patients than in those from healthy volunteers (table 1) by real-time RT-PCR (fig. 1b).

Table 2. List of selected genes by GeneChip analysis

Comparison analysis	Number of genes	Probe set	Accession No.	Annotation	Differential call	Fold change
Mild/healthy	8	36068_at	AF002210	copper chaperone for SOD	increase	4.0
		36647_at	AA526812	NCI CGAP Pr2 EST	increase	3.3
		37383_f_at	X58536	HLA class I heavy chain locus C	decrease	−4.7
		41577_at	AB020630	KIAA0823	increase	3.7
		1353_g_at	U11870	IL-8 receptor type A	decrease	−14.5
		32814_at	M24594	interferon-inducible 56-kDa protein	decrease	−6.9
		34230_r_at	D84454	UDP-galactose translocator	increase	3.5
		36031_at	AB024401	p33	increase	4.0
Severe/healthy	13	280_g_at	L13740	Nur77	increase	4.5
		33319_at	AF009674	AXUN	increase	7.0
		34325_at	AJ005893	JM26 protein	increase	3.2
		35083_at	AL031670	ferritin, light polypeptide-like 1	decrease	−3.7
		35297_at	AC002400	chromosome 16 BAC clone CIT987SK-A-735G6	increase	3.2
		36113_s_at	AJ011712	TNNT1 gene, exon 1–11	increase	9.1
		36674_at	J04130	Act-2	increase	8.5
		38599_s_at	AD001530	XAP-5	increase	5.9
		38976_at	D44497	actin binding protein p57	decrease	−3.3
		39780_at	M29551	calcineurin A2	increase	8.0
		40375_at	X63741	pilot mRNA	increase	6.6
		41163_at	AL109672	cDNA clone EUROIMAGE 755868	increase	4.4
		676_g_at	J04164	interferon-inducible protein 9–27	decrease	−4.3
Severe/mild	3	40659_at	U12767	NOR1	increase	4.6
		38994_at	AF037989	STAT-induced STAT inhibitor-2	increase	4.0
		34449_at	U13022	negative regulator of programmed cell death ICH-1S	decrease	−3.7

Among peripheral blood leukocyte fractions from 5 healthy volunteers, the basal NOR1 expression level was dominant in eosinophils. The neutrophil, B cell, T cell, and monocyte fractions showed little NOR1 expression. However, the expression of genes of the other NR4A family members, Nur77 and Nurr1, was not eosinophil specific in the leukocyte fractions (fig. 2).

Expression of NR4A Nuclear Receptor Family Genes in Cultured Eosinophils or the Eosinophil Cell Line, AML14.3D10

The expression mechanism of the NR4A nuclear receptor family genes was studied in vitro using cultured eosinophils isolated from healthy volunteers. After dose-related stimulation by cytokines, such as IL-4, IFN-γ, GM-CSF, IL-3, eotaxin, or IL-5, there was no increase in the gene expression. Similar negative results were seen after steroid treatment (data not shown).

We have previously reported that anti-CD30 Ab and anti-Fas Ab induced eosinophil apoptosis in the presence of IL-5 [23]. In this study, Fas Ab-induced apoptosis was not eosinophil specific. Rapid expression of the NR4A receptor family, especially Nur77 and NOR1, was observed with the anti-CD30 Ab treatment, but not with the anti-Fas Ab, in cultured peripheral blood eosinophils after 1–3 h (fig. 3). The rapid NR4A orphan nuclear receptor family gene expression and the subsequent apoptosis after soluble anti-CD30 Ab stimulation were also observed in the eosinophil cell line, AML14.3D10 (fig. 4). In this cell line, Nur77 and NOR1 were also rapidly expressed in both the presence (data not shown) and absence of IL-5. Induction of the Nurr1 gene expression was not observed clearly. Both the apoptosis of the AML14.3D10 cell line and NR4A gene family expression was downregulated by the MAPK inhibitor, U0126 (fig. 5). However, the p38 inhibitor, SB203508, had no effect on either the NR4A family gene expression or the subsequent apoptosis (fig. 5).

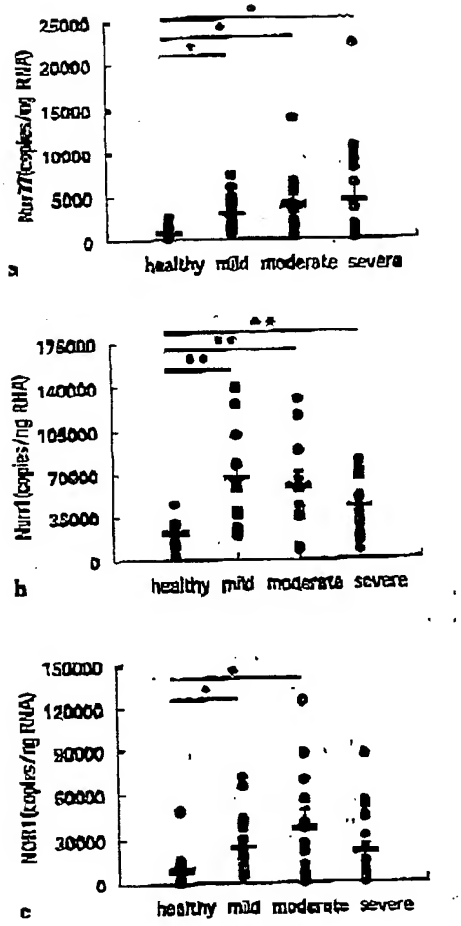

Fig. 1. The expression levels of NR4A nuclear receptor family mRNA in peripheral blood eosinophils. The expression of three nuclear receptor genes, Nur77 (a), Nurr1 (b), and NOR1 (c), in eosinophil samples from healthy volunteers and patients with mild, moderate, and severe AD is shown (mean ± SE, * $p < 0.05$, ** $p < 0.01$). The copy numbers of each transcript per 1 ng RNA, standardized by levels of the GAPDH transcript, are shown on the ordinate.

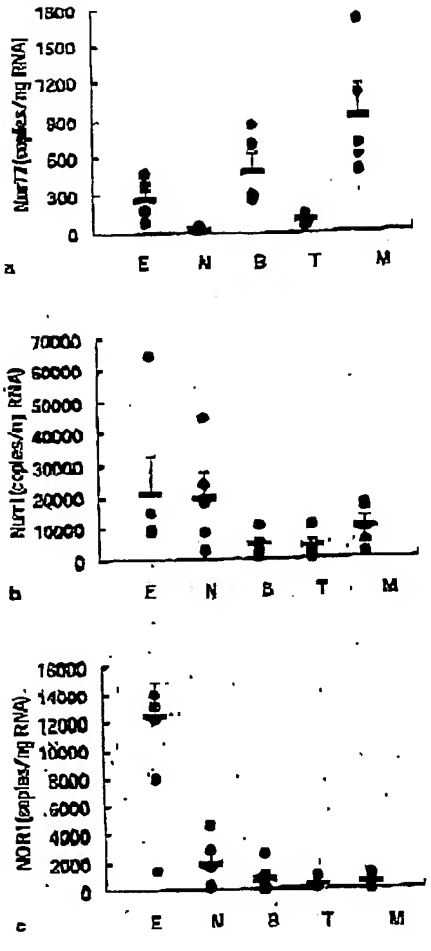

Fig. 2. The expression levels of NR4A nuclear receptor family mRNA in peripheral blood leukocyte fractions. The expression of three nuclear receptor genes, Nur77 (a), Nurr1 (b), and NOR1 (c), in eosinophil (E), neutrophil (N), B cell (B), T cell (T), and monocyte (M) fractions of 5 healthy volunteers is shown (mean ± SE). The copy numbers of each transcript per 1 ng RNA, standardized by levels of the GAPDH transcript, are shown on the ordinate.

Discussion

We have identified genes that are expressed at significantly higher levels in eosinophils from AD patients when compared to eosinophils from healthy volunteers. Such information will help to better understand the pathogenesis of allergic diseases such as AD. It was surprising that two genes in the same nuclear receptor family were identified out of the relatively small number of genes that were selected by comprehensive expression analysis of 12,626 genes. Nuclear receptors are implicated in many human diseases and one of the target molecules for which drug treatment can likely be found [30]. Our expression pattern data point to the pathological importance of these receptor family genes in AD conditions. The finding that Nurr1, another member of the NR4A receptor family, was ex-

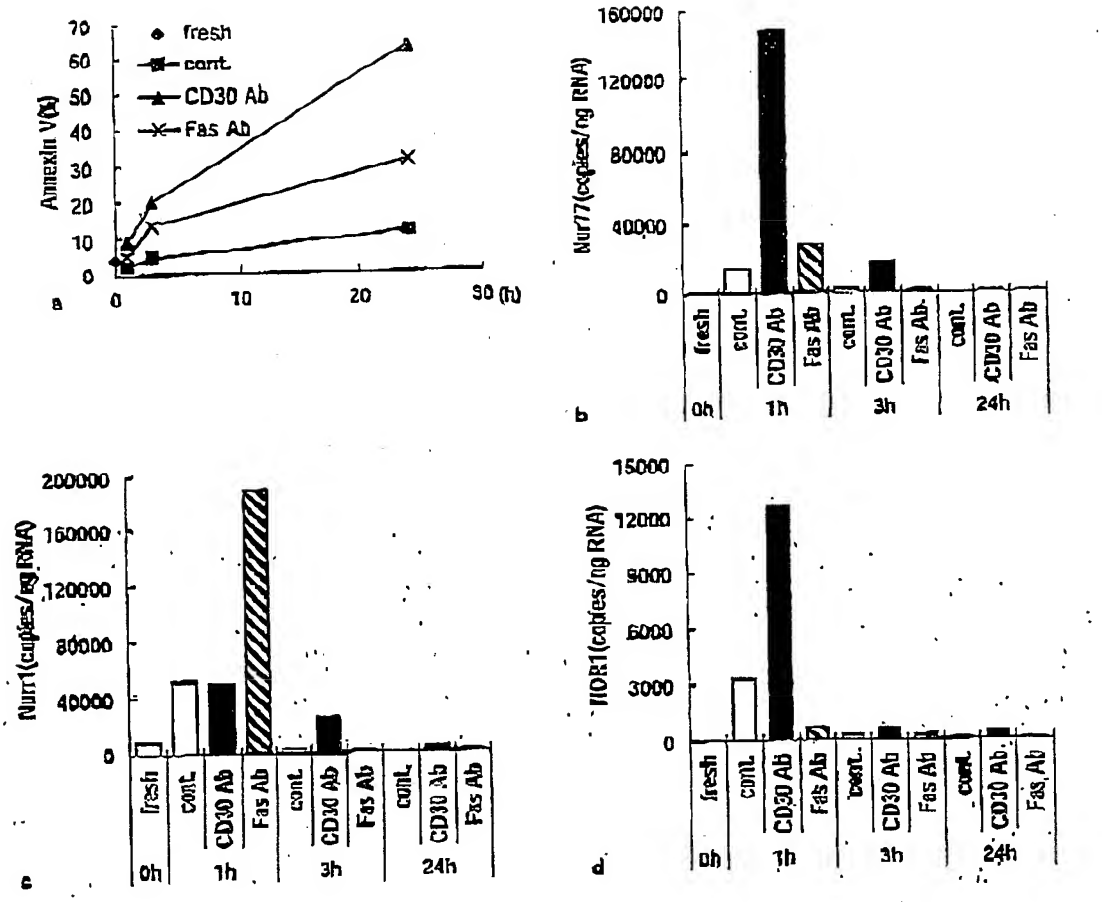

Fig. 3. Apoptosis induction and the expression levels of NR4A nuclear receptor family mRNA in peripheral blood eosinophils of healthy volunteers after anti-CD30 or anti-Fas Ab stimulation. The in vitro time course of apoptosis (a) and nuclear receptor gene expression for Nur77 (b), Nurr1 (c), and NOR1 (d) in peripheral blood eosinophils in the presence of 1 ng human recombinant IL-5 are shown. The copy numbers of each transcript per 1 ng RNA, standardized by levels of the GAPDH transcript, are shown on the ordinate.

pressed at a higher level in eosinophils of AD patients than in eosinophils of healthy volunteers reinforces this conclusion. The enhancement of NR4A receptor gene expression in eosinophils correlated well with the increase in numbers of eosinophils in peripheral blood in previously documented patient groups [26]. The AD patients were not age matched in this study to the healthy volunteer group. However, age differences are unlikely to account for the differential expression between AD patients and healthy volunteers because both younger and older AD patients have been reported to have similar responses [31, 32].

The findings that only NOR1 expression was eosinophil specific when compared with other leukocyte fractions, as well as patterns of the expression of Nur77 and Nurr1 in the same fractions suggest that NOR1 expression is the most important of the NR4A nuclear receptor family genes in AD pathology. Differences in NOR1 gene expression were also found by comparisons of differential display analysis of eosinophils in identical patients during both the exacerbation and remission phases of AD. NOR1 expression was remarkably elevated by bedside treatment and was accompanied by a drastic decrease in

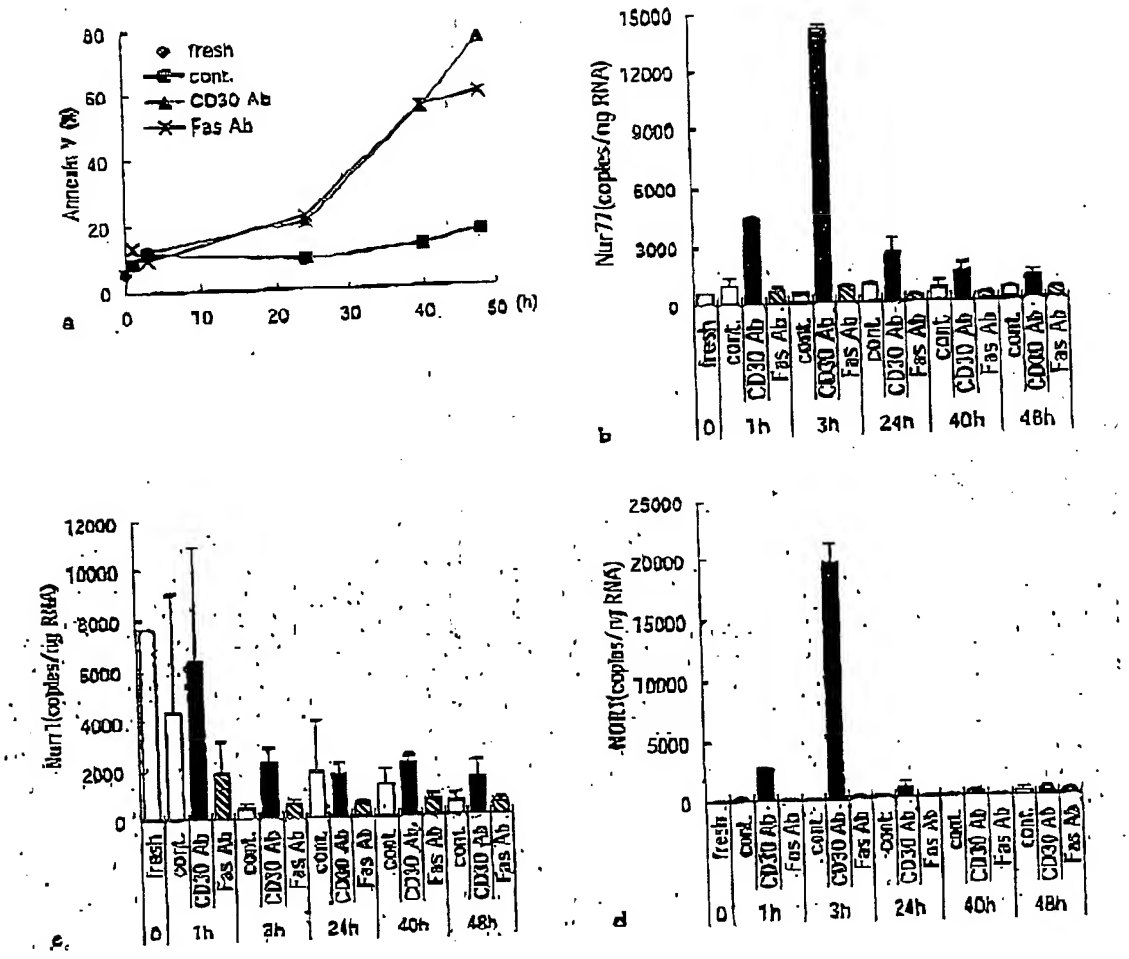

Fig. 4. Apoptosis induction and the expression levels of NR4A nuclear receptor family mRNA in AML14.3D10 after anti-CD30 or anti-Fas Ab stimulation. The in vitro time course of apoptosis (a) and nuclear receptor gene expression for Nur77 (b), Nurr1 (c), and NOR1 (d) in the eosinophil cell line in the absence of human recombinant IL-5 are shown as mean ± SE. The copy numbers of each transcript per 1 ng RNA, standardized by levels of the GAPDH transcript, are shown on the ordinate.

the number of eosinophils in peripheral blood (data not shown). The changes in the expression of NR4A nuclear receptor genes, which correlates with the number of eosinophils in pathological conditions, indicates that these genes may be related to eosinophil apoptosis. Nur77 and NOR1 have been reported to be apoptotic genes in T cells [19–22]. Activation-induced cell death signaling in T cells through T cell receptors dramatically induces these two genes (data not shown).

Clarification of the functional roles of NR4A nuclear receptors is essential because expression analysis cannot determine whether the differential expression has a causative effect on disease or whether it is linked to the expression of other genes important in disease. To explore these two possibilities, we used an in vitro eosinophil culture system and tested the effect of various stimulation conditions. We measured the expression of many eosinophil genes from healthy volunteers after stimulation with cytokines, such as IFN-γ, GM-CSF, IL-3, cotaxin or IL-5 in both a time- and dose-dependent manner. It has been reported that IL-5, IL-3, and GM-CSF prolong eosinophil survival and inhibit apoptosis [33–35]. In contrast, IL-4

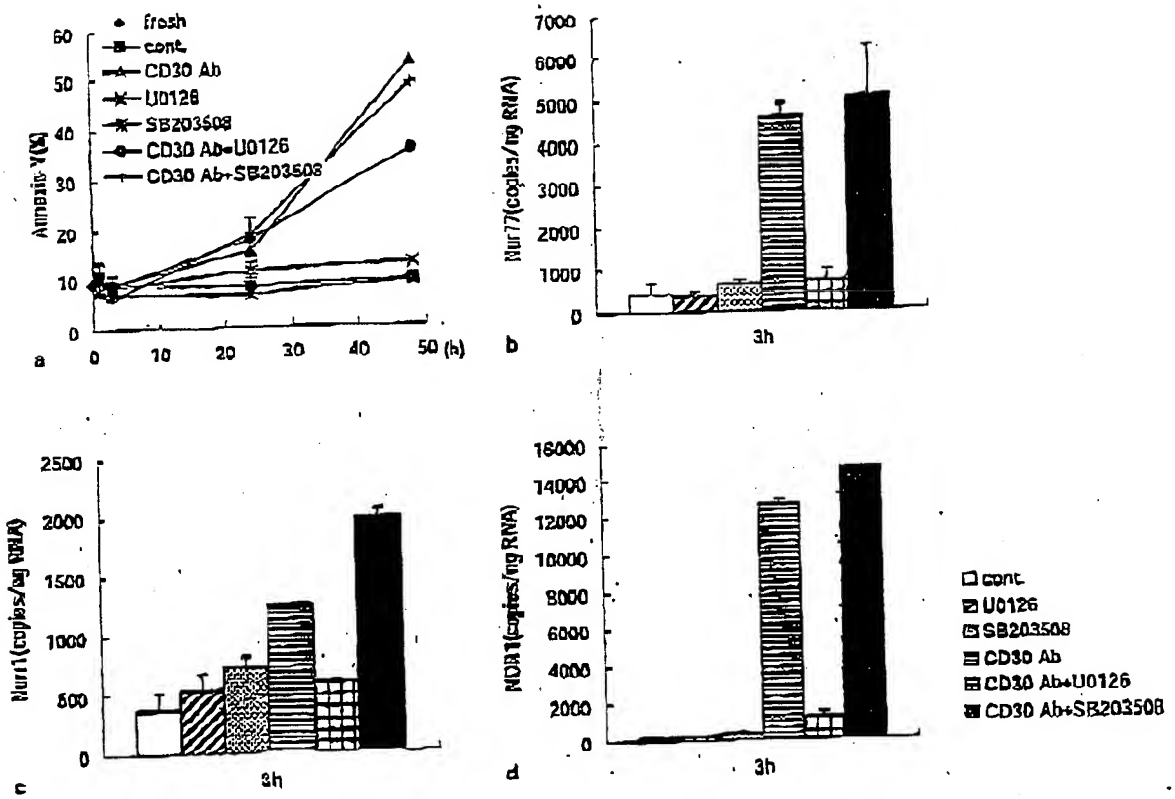

Fig. 5. Effect of the signal transduction inhibitors on anti-CD30 Ab-induced apoptosis and the expression of NR4A nuclear receptor family mRNA in AML14.3D10. Inhibition of apoptosis (**a**) and nuclear receptor gene expression for Nur77 (**b**), Nurr1 (**c**), and NOR1 (**d**) are shown. Inhibition time course of apoptosis and inhibition of the gene expression by U0126 and SB203508 after 3 h incubation are indicated as mean ± SE. The copy numbers of each transcript per 1 ng RNA, standardized by levels of the GAPDH transcript, are shown on the ordinate.

induces apoptosis in peripheral blood eosinophils [36]. Several genes, including those for cytokine receptors and antiapoptotic genes, such as bcl-2 and bcl-xL, were highly expressed in eosinophils from both AD patients and after IL-5 and IL-4 stimulation [26]. However, the relationships between these genes and NR4A receptor family genes are unknown because stimulation with cytokines had no effect on the expression of any of the NR4A receptor genes in eosinophils. Steroids are possible inducers of eosinophil apoptosis [37–39]. However, in our study, steroids had no effect on the expression of NR4A genes in a leukocyte population that included eosinophils (data not shown). Therefore, differential expression of NR4A nuclear receptor gene in clinical samples of eosinophils from AD patients is likely to be independent of the nonsystemic steroid treatment.

We have found that eosinophil-specific unique apoptosis signaling occurs through CD30 molecules [23]. A positive correlation between the level of soluble CD30 molecule and the serum eosinophil cationic protein levels in patients with AD indicates the essential nature of CD30-mediated signaling to the pathology of AD [40, 41]. Soluble CD30 can inhibit CD30-CD30 ligand signals in vivo [42]. Rapid induction of Nur77 and NOR1 receptors 1–3 h after agonistic plate-bound anti-CD30 Ab stimulation of cultured peripheral blood eosinophils suggests that events different from those involved in Fas signaling result in eosinophil-specific apoptosis. The similar results

in the eosinophil cell line, AML14.3D10, for a scarce induction of Nurr1 support the likelihood of distinct pathways. The experiment using specific signal transduction inhibitors also confirmed the relation between eosinophil-specific apoptosis and rapid NR4A nuclear receptor gene expression. Both the apoptosis of the eosinophil cell line and rapid expression of the NR4A nuclear receptor family were downregulated by the MAPK inhibitor. Therefore, ERK1/2 phosphorylation by MEK1/2 existing downstream of CD30 signaling and upstream of the nuclear receptor family could be involved in eosinophil apoptosis via expression regulation of the NR4A nuclear receptor family genes in allergic conditions such as AD. p38 phosphorylation regulation does not appear to have an impact on either the receptor family gene expression or the subsequent apoptosis.

The expression of the CD30 molecule is consistently detected at low levels on eosinophil surfaces [23] and transmits signals through adaptor proteins of the TNFR-associated factor (TRAF) family, including TRAF-1, -2, and -5. The result of CD30 signaling is the activation of transcription factors such as NF-kB and Jun N-terminal kinase [43–47]. We can now add the NR4A receptor family, one of the families of genes for transcription factors, as the source of molecules which intervene in the CD30-mediated activation pathway.

Since NR4A nuclear receptor family genes are induced at immediate-early times in many tissues, the gene family may also be involved in other functions besides apoptosis [48]. In this paper, we have reported that the NR4A nuclear receptor family genes are abundantly expressed in human peripheral eosinophils in AD conditions, and we suggest that they play specific roles in eosinophil-specific apoptosis. NR4A nuclear receptor family genes encode orphan receptors [30]. Either yet-to-be discovered ligands or specific compounds that regulate Nur77-, Nurr1-, or NOR1-dependent transcriptional activity are potentially valuable pharmaceutical treatments for AD since they are likely to regulate eosinophil apoptosis. Our hope is that these results will encourage the search for new therapeutic targets that are directed to eosinophils for treatments of allergic diseases such as AD.

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U.S. PATENT DOCUMENTS

EXAMINER INITIAL	REF. NO.	DOCUMENT NUMBER Number-Kind Code (if known)	ISSUE DATE / PUBLICATION DATE MM-DD-YYYY	NAME OF PATENTEE OR APPLICANT OF CITED DOCUMENT
	AA	US 2002/0049151 A1	04/25/2002	Murphy, *et al.*
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	AM	EP 1287019 A1	11/22/2001	Baylor College of Medicine		
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	AQ	EP 1185647 A1	12/21/2000	Smith Kline Beecham PLC		
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